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Characterization of antigenicity and immunogenicity patterns of native and recombinant zona pellucida proteins in the white-tailed deer (*Odocoileus virginianus*)

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The effectiveness of zona pellucida antigens in immunizing white-tailed deer to reduce fertility was evaluated by analysing the constituent deer zona pellucida proteins and their immunogenicity. Does were immunized with porcine zona pellucida antigens. The antibodies were characterized using immunohistochemical and immunoblot analysis, in which zona pellucida proteins were separated by one-dimensional and two-dimensional PAGE. Deer anti-porcine zona pellucida antibodies were found to recognize all the major proteins of the porcine zona pellucida. These antibodies also recognized several proteins of deer zona pellucida, indicating that it is possible to break immune tolerance in the deer using such a protocol. The antibodies were also found to recognize peptides of 55 and 75 kDa that were produced by expressing cDNA clones containing antigens of major glycoproteins of rabbit zona pellucida. Furthermore, antibodies against rabbit zonae pellucidae recognized antigens in zonae of paraffin-embedded deer ovaries. Taken together, these experiments demonstrate the crossreactive nature of a number of zona pellucida epitopes found in deer and in several other species. They also illustrate the immunogenicity possible in such an immunization protocol, and provide valuable probes for the investigation of follicular development in this and other species.

Introduction

The population of white-tailed deer in the USA has continued to rise. This problem has resulted in the need for effective and humane reduction of these numbers. The development of antifertility vaccines for animal populations has become increasingly important in the past few years because these methods could provide inexpensive and effective population control alternatives. The proposed vaccines, administered actively or passively, target a variety of antigens such as hormones (Griffin, 1983; Moudgal, 1983; Raj, 1983; Thau *et al.*, 1986), or gametes (Alexander and Isahakia, 1983; Anderson, 1983; Goldberg and Shelton, 1983; Goldberg *et al.*, 1983; Mahi-Brown *et al.*, 1983; Menge, 1983; Sacco *et al.*, 1983a, b; Skinner *et al.*, 1984). While the development of a contraceptive vaccine for humans involves safety, effectiveness and potential reversibility, the criteria for wildlife management may be different. In some animal populations, it is desirable (if not required) that animals are permanently sterilized (Dunbar and Prasad, 1993). In other populations, it is desirable to have a reversible, safe and cost-effective vaccine. Several strategies will probably be necessary to develop optimal vaccines for different animal species.

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To date, the majority of studies on contraceptive vaccines targeted to the female gamete have used antigens associated with the zona pellucida, which not only protects the egg and developing embryo, but is also critical for the early stages of growth and development of the egg in the ovarian follicle. It is also important in the fertilization process, as the spermatozoa must bind to and penetrate it before encountering the egg vestment. The antigens of the zona pellucida have long been considered attractive targets for an immunocontraceptive vaccine (Wolgemuth *et al.*, 1984; Dunbar *et al.*, 1989b; Yurewicz *et al.*, 1993). The development of procedures to isolate large numbers of oocytes within their zonae pellucidae has made it possible to study the glycoprotein antigens of the zona pellucida in great detail (Sacco *et al.*, 1981a, b, 1983b; Skinner *et al.*, 1984; Skinner and Dunbar, 1986; O'Rand, 1988; Timmons and Dunbar, 1988; Wassarman, 1988; Dunbar *et al.*, 1989a).

It has been further demonstrated that it is impossible to predict the immune response of an individual species when it is immunized with zona pellucida antigens of a different species (Dunbar *et al.*, 1989b, 1991; Timmons *et al.*, 1990; Schwoebel *et al.*, 1992). A significant observation was that alloimmunization with zona pellucida glycoproteins of the same species does not elicit a significant immune response, whereas heteroimmunization with the zona pellucida glycoproteins of a

different mammal elicits a dramatic immune response, including the production of antibodies that recognize the 'self' determinants of the zona pellucida (Maresh and Dunbar, 1987; Skinner *et al.*, 1987). The immunogenicity of zona pellucida glycoproteins is therefore primarily due to the foreign epitopes associated with the zona pellucida of different species. However, the biological effectiveness of the vaccine is provided by the antibodies directed against epitopes that are shared among different mammalian species. Taking these observations into account, we have used genetic engineering techniques to develop specific strategies for dissecting specific domains of the zona pellucida molecules that are critical for eliciting a significant immune response (i.e. enhanced immunogenicity) from those that are critical for recognition by antibodies (i.e. antigenicity) and that inhibit fertilization or oocyte and ovarian follicular development (Schwoebel *et al.*, 1991; 1992; Lee and Dunbar, 1993).

Materials and Methods

Animal immunization and production of antibodies

Three captive adult does (white-tailed deer: *Oidocoileus virginianus*) were immunized with heat-solubilized porcine zonae pellucidae (HSPZ) (300 μg per animal) emulsified in 1 ml saline: complete Freund's adjuvant (1:1) by i.d. injection (30–40 μl at a minimum of 3–4 sites) at the Pennsylvania State University facility, University Park, PA. Boosters containing 150 mg of HSPZ were given s.c. 4, 8 and 12 weeks afterwards. Routes of administration were those that had been found to be most effective in smaller animals (Dunbar and Raynor, 1980; Wood *et al.*, 1981). Blood was collected via the jugular vein, and the sera from each animal frozen at −20°C. Two other adult does supplying tissue for immunohistochemistry were killed with an overdose of sodium pentobarbital and the ovaries immediately removed and fixed.

Six rabbits were immunized with HSPZ that had been isolated as previously described, and their sera pooled and tested to verify a high titre of anti-zona pellucida antibodies (Skinner *et al.*, 1984; Dunbar, 1987). The serum pool was kept frozen at −20°C until required. Two additional rabbits were immunized (using the above protocol) with zonae pellucidae that had been isolated from frozen deer ovaries (see below); the sera of the animal that gave the most intense response against heat-solubilized deer zonae pellucidae (HSDZ) on immunoblotting was used in these studies. Antisera were collected and kept frozen at −20°C until required.

Antisera against heat-solubilized rabbit zonae pellucidae (HSRZ) and against rabbit zona pellucida proteins (see below) were generated in guinea-pigs by methods (equivalent to those for deer and rabbits) described in detail by Lee and Dunbar (1993). Blood samples were collected from guinea-pigs using cardiac puncture under general anaesthesia (42.8 mg ketamine ml^{-1}, 8.6 mg xylazine ml^{-1} and 1.4 mg acepromazine ml^{-1} given i.m. at a dosage of 0.5–0.7 ml kg^{-1}). Antisera were stored frozen at −20°C until required.

Ethics of experimentation

Immunization and tissue and blood sample collection from does were undertaken with the approval of the Pennsylvania State University Institutional Animal Care and Use Committee. Rabbits and guinea-pigs were immunized, sampled for blood and killed following the guidelines of the Baylor College of Medicine Institutional Review Board.

Isolation and solubilization of zona pellucida protein

Zonae pellucidae were isolated, solubilized and used as immunogens for antibody production or as antigens for antibody testing. Pig ovaries were obtained frozen from abattoirs, and rabbit ovaries were obtained from PelFreez Biological (Rogers, AR). Deer were killed at the Pennsylvania State University, and ovaries removed and immediately frozen in liquid nitrogen. Alternatively, ovaries were collected post-mortem by authorized US Department of Agriculture personnel. The isolation of pig and rabbit zonae pellucidae was carried out as described by Wood *et al.* (1981). Deer zonae pellucidae were isolated under a dissecting microscope; approximately 2000 zonae pellucidae may be retrieved from 20 deer ovaries in this way. The zona pellucida glycoproteins from all species were solubilized by heating them for 1 h at 68°C in 0.4 mol sodium carbonate l^{-1}, pH 9.5. After pelleting cellular debris by centrifugation at 12 000 *g* for 10 min, the supernatant was collected and protein content was determined by the method of Lowry *et al.* (1951).

Treatment of zona pellucida glycoproteins with endo-β-galactosidase (EBGD)

Partial deglycosylation of rabbit or pig zona pellucida glycoproteins was accomplished by treating them with endo-β-galactosidase (Miles Scientific, Naperville, IL) (Fukuda, 1976). Briefly, 100 μg of HSRZ or HSPZ glycoprotein was incubated for 18 h in the presence of 0.1 mol sodium acetate buffer l^{-1}, pH 5.8, and 0.001 U enzyme. The reaction mixture was quick-frozen to stop the reaction, and lyophilized to 100 μl. An equal volume of electrophoresis solubilization buffer was added and the solution was boiled for 10 min, and the resulting partially deglycosylated zona pellucida proteins were resolved by one-dimensional or two-dimensional PAGE. When HSRZ proteins were resolved by two-dimensional PAGE, species of 45, 55 and 75 kDa were observed. The 45 and 55 kDa proteins were stained with Coomassie blue, excised from the gels and used as immunogens as described below.

Production of recombinant rabbit zona pellucida proteins

The pEX plasmids were used to express the recombinant proteins rec55, rec75a and rec75b, as described in detail by Stanley and Luzio (1984), Schwoebel *et al.* (1991), and Lee and Dunbar (1993). These samples were subjected to one-dimensional PAGE for immunoprobing with deer anti-HSPZ antisera from several animals.

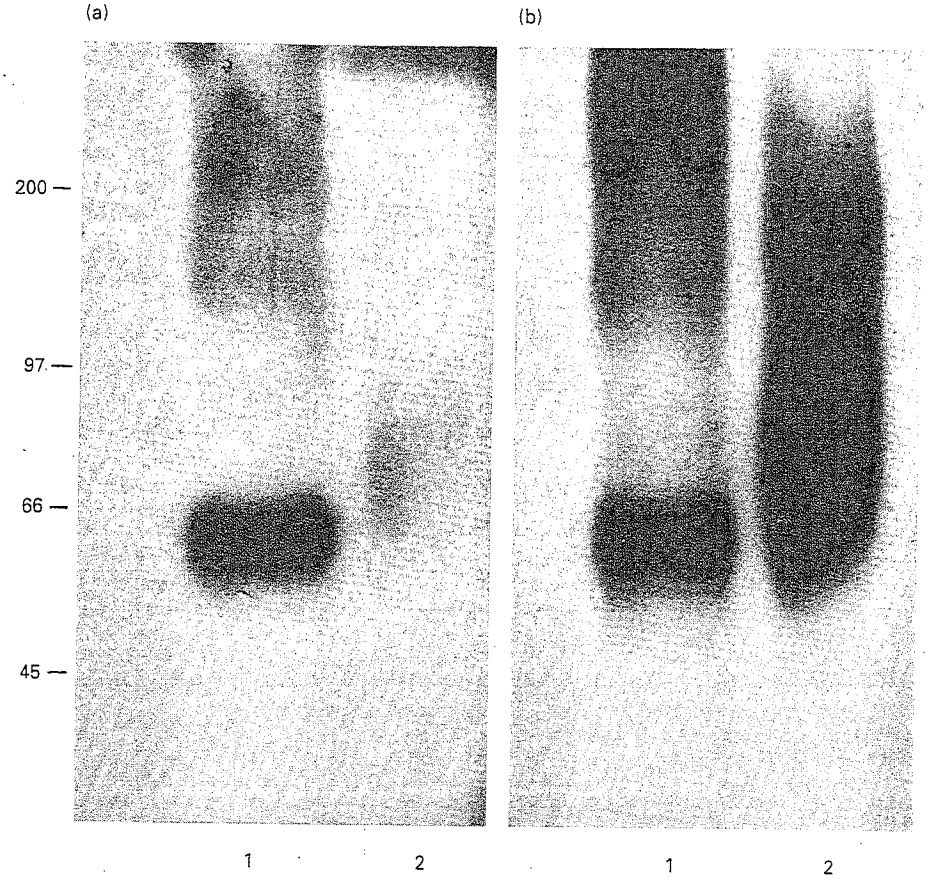

Fig. 1. Autoradiographs showing electroblotted samples of partially deglycosylated rabbit zona pellucida (lane 1) and pig zona pellucida (lane 2) separated on 7.5% one-dimensional PAGE. The proteins were (a) probed with rabbit anti-deer zona pellucida antiserum and the same blot was (b) subsequently re-probed with rabbit anti-heat-solubilized porcine zonae pellucidae antiserum to demonstrate complete electroblot transfer of both sets of zona proteins. Antibodies were detected with ^{125}I-labelled Protein A.

Gel electrophoresis and immunoblotting

One-dimensional PAGE was conducted according to Laemmli (1970). Two-dimensional PAGE was accomplished as described by Dunbar (1987). Transfer was completed in 40 min using the buffer described by Matsudaira (1987): 10 mmol CAPS l^{-1}, 10% methanol, pH 10.0. After transfer of the protein from the gel to the membrane, the immunoblot was processed as described by Dunbar (1987). Briefly, the membranes were blocked in a Tris-buffered saline–5% milk solution and probed with the appropriate antiserum or cross-species, affinity-purified antibody solution (1:100). Where necessary (as with deer antisera), a secondary antibody [goat anti-deer immunoglobulin G (IgG); 1:100] (Cappel, Durham, NC) and a tertiary antibody (rabbit anti-sheep IgG; 1:500) (Boehringer-Mannheim, Indianapolis, IN) were used, and the antibody sandwich then visualized using (1.0 μCi ^{125}I-labelled protein A ml^{-1}) (ICN, Irvine, CA) and autoradiography.

Affinity purification of antisera

Guinea-pig antisera containing antibodies against 55 and 45 kDa rabbit zona proteins were affinity-purified across species on a rabbit zona pellucida column to select for those antibody populations that recognized epitopes common to both guinea-pigs and rabbits (Skinner *et al.*, 1987). Antibodies purified in this way were then used in immunocytochemistry studies.

Histology and immunocytochemistry

Deer ovaries were collected after killing untreated does at the Pennsylvania State University facility, and fixed in Bouin's fluid. Tissues were then embedded in paraffin wax, sectioned and stained in periodic acid Schiff stain by routine methods. Corresponding serial sections were mounted unstained and submitted to immunocytochemical localization studies, as described by Lee and Dunbar (1993). Briefly, sections were blocked in 0.1% BSA for 20 min and treated overnight at 4°C with the primary guinea-pig or rabbit antibody solutions (1:500) in a moist chamber. The sections were rinsed in 0.1% BSA and treated with biotinylated rabbit anti-guinea-pig IgG (1:200) (Vector Laboratories, Burlingame, CA) or biotinylated goat anti-rabbit IgG (1:200) (Vector Laboratories). This was followed by treatment with the peroxidase-labelled avidin–biotin complex reagent (Vector Laboratories); colour was

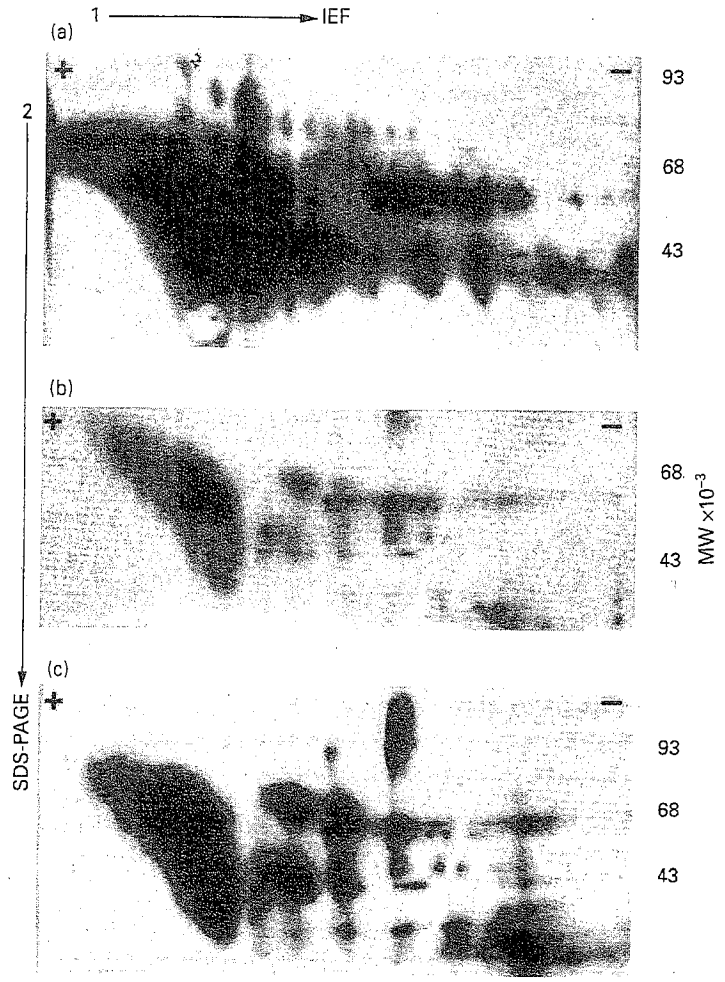

Fig. 2. Autoradiographs of two-dimensional PAGE immunoblots of zona pellucida glycoproteins. (a) Porcine zonae pellucidae probed with deer anti-heat-solubilized porcine zonae pellucidae (HSPZ) antiserum is detected by goat anti-deer immunoglobulin, rabbit anti-sheep immunoglobulin, and ^{125}I-labelled Protein A. (b) Deer zonae pellucidae probed with deer anti-HSPZ antiserum used in (a). (c) Immunoblot shown in (b) re-probed with rabbit anti-HSPZ antisera detected by ^{125}I-labelled Protein A.

developed using 0.05% diaminobenzidine hydrochloride as chromophore.

Results

Characterization of deer zona pellucida glycoproteins

Rabbits were immunized with HSDZ, and antiserum from one of these animals used to probe deglycosylated rabbit and porcine zonae on a one-dimensional PAGE immunoblot. This antiserum was reactive with bands in the lane containing rabbit zona pellucida but not in that containing porcine zona pellucida (Fig. 1a). When this blot was subsequently re-probed with antiserum made in rabbit against native HSPZ, the porcine zona pellucida did transfer during the electroblotting procedure, but was not then recognized by the rabbit anti-deer zona pellucida antiserum under these conditions (Fig. 1b). However, when native porcine zona pellucida was subjected to chromatography using two-dimensional PAGE, immunoblotted on to a polyvinylidene fluoride membrane, and probed with deer anti-HSPZ antiserum, subsequent autoradiography (Fig. 2a) showed that the deer immune system recognizes all three of the well-known major pig zona glycoproteins.

Characterization of deer autoimmune response to deer zona pellucida antigens

Deer zonae pellucidae (approximately 1000 zonae pellucidae per gel) were solubilized and submitted to two-dimensional PAGE, immunoblotting and probing with the same deer anti-HSPZ antiserum. The autoradiograph shown in Fig. 2b indicates that the immune system of the deer recognizes several major deer zona pellucida antigens, demonstrating the breaking of immune tolerance by this cross-immunization protocol. A subsequent re-probe of this same blot with rabbit

anti-HSPZ serum (Fig. 2c) established the species crossreactive nature of this system, as anti-porcine zona pellucida antibodies formed in the rabbit recognize further antigenic elements in the deer zona pellucida glycoprotein complex.

It should be noted that secondary nonspecific reactions of the goat anti-deer secondary antibody and the ^{125}I-labelled Protein A reagent with non-zona pellucida elements occurred in the two-dimensional PAGE preparation of deer zonae pellucidae. However, this is an inevitable result of the solubilization of small numbers of deer zonae pellucidae isolated with difficulty from scarce deer ovaries. Such a protocol must emphasize retention of all available zonae at the expense of rigorous isolation. Hence, some contaminant proteins are always present in the deer zona pellucida preparation, which is subsequently detected by two-dimensional PAGE. Comparison of Fig. 2c with Fig. 2a shows the level of purification that can be achieved with large numbers of zonae (available with pig ovaries) and extensive isolation before electrophoretic separation. Nevertheless, the major deer zona pellucida glycoproteins are easily characterized by immunoblot procedures, and their recognition by both deer and rabbit anti-zona antibody populations are obvious.

Shared antigens on recombinant zona proteins

Figure 3 shows the recognition response of antisera from three different HSPZ-immunized deer to recombinant rabbit zona proteins immunoblotted from one-dimensional PAGE gels. The autoradiographed blots show recognition by the deer immune system not only of native HSPZ, but also of antigenic determinants held in common among all three rabbit clones. While the intensity of the antibody response to these antigens varies among the three deer, there is no significant recognition of native HSRZ or of any bacterial contaminants.

Immunocytochemical localization within the deer ovary

A panel of polyclonal antibodies made in guinea-pigs against rabbit zonae pellucidae and against two of their deglycosylated protein components was used to probe paraffin wax embedded samples of adult deer ovaries. The histology of portions of this ovary in sections stained with periodic acid Schiff containing a growing follicle and an antral follicle are shown (Fig. 4a and b, respectively.) The thin, delicate structure of the deer zona pellucida (Fig. 4a) is unusual among mammals. A more eccentric section (Fig. 4b) through an older follicle gives an impression of a thicker zona, and shows the cumulus granulosa cells surrounding the egg in the antral follicle. Figure 4c and d show two views of the cumulus oophorus mass of granulosa cells surrounding the antral deer follicle that have been probed with a purified antibody against a deglycosylated major rabbit zona pellucida protein (55 kDa). An eccentric section (Fig. 4c) shows a small number of reactive granulosa cells in the cumulus mass. A more central section (Fig. 4d) indicates the presence of the antigenic determinant in the zona pellucida and in a few of the surrounding granulosa cells. Figure 4e and f show serial sections corresponding to that in Fig. 4d in which two other antibody probes, anti-rabbit 45 kDa protein and anti-HSRZ, give similar results.

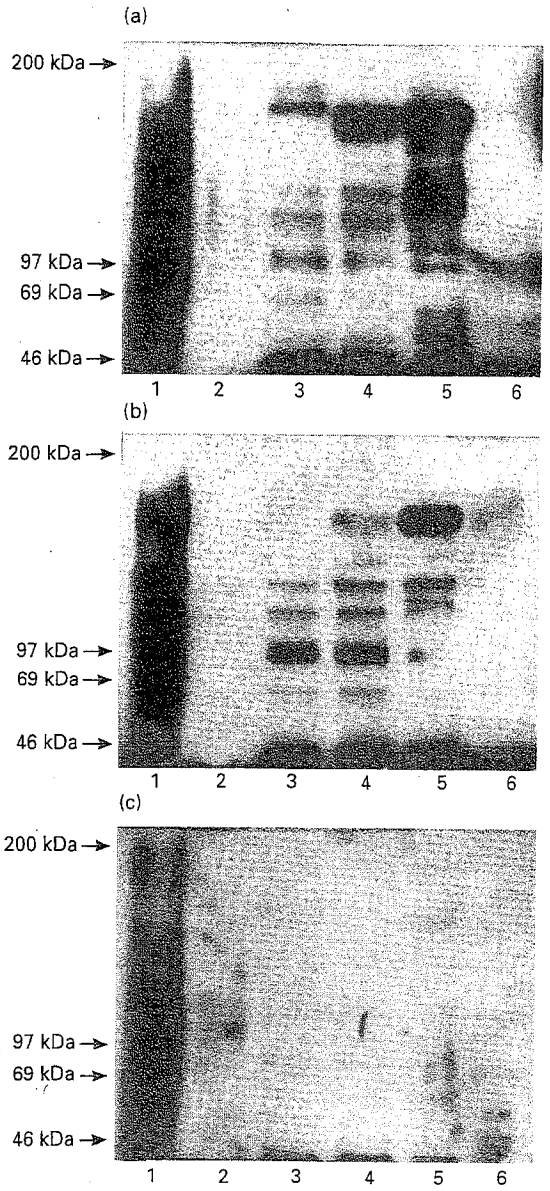

Fig. 3. Autoradiographs of immunoblots showing that deer anti-heat-solubilized porcine zonae pellucidae antisera from three different immunized deer (a,b,c) recognize native and recombinant zona pellucida proteins separated by one-dimensional PAGE gels and transferred on to a PVDF membrane. Lane 1: control antigen HSPZ; lane 2: control antigen heat-solubilized rabbit zona pellucida; lane 3: recombinant rabbit 55 kDa zona pellucida protein (rec55); lanes 4, 5: recombinant peptides (rec75a and 75b, respectively) of the 75 kDa rabbit zona pellucida protein; lane 6: cro-β-galactosidase (negative control) protein. Primary antisera probes were detected by goat anti-deer immunoglobulin, rabbit anti-sheep immunoglobulin and ^{125}I-labelled Protein A.

In addition, antibodies present in sera of rabbits immunized against HSDZ were used in immunocytochemical localization experiments on adult deer ovaries to probe follicles at a range of growth stages. These antibodies are compared with those raised in guinea-pigs against HSRZ (Fig. 5). Again, the epitopes shared between rabbit and deer species are evident. The follicles range from primary follicles to antral follicles, and the

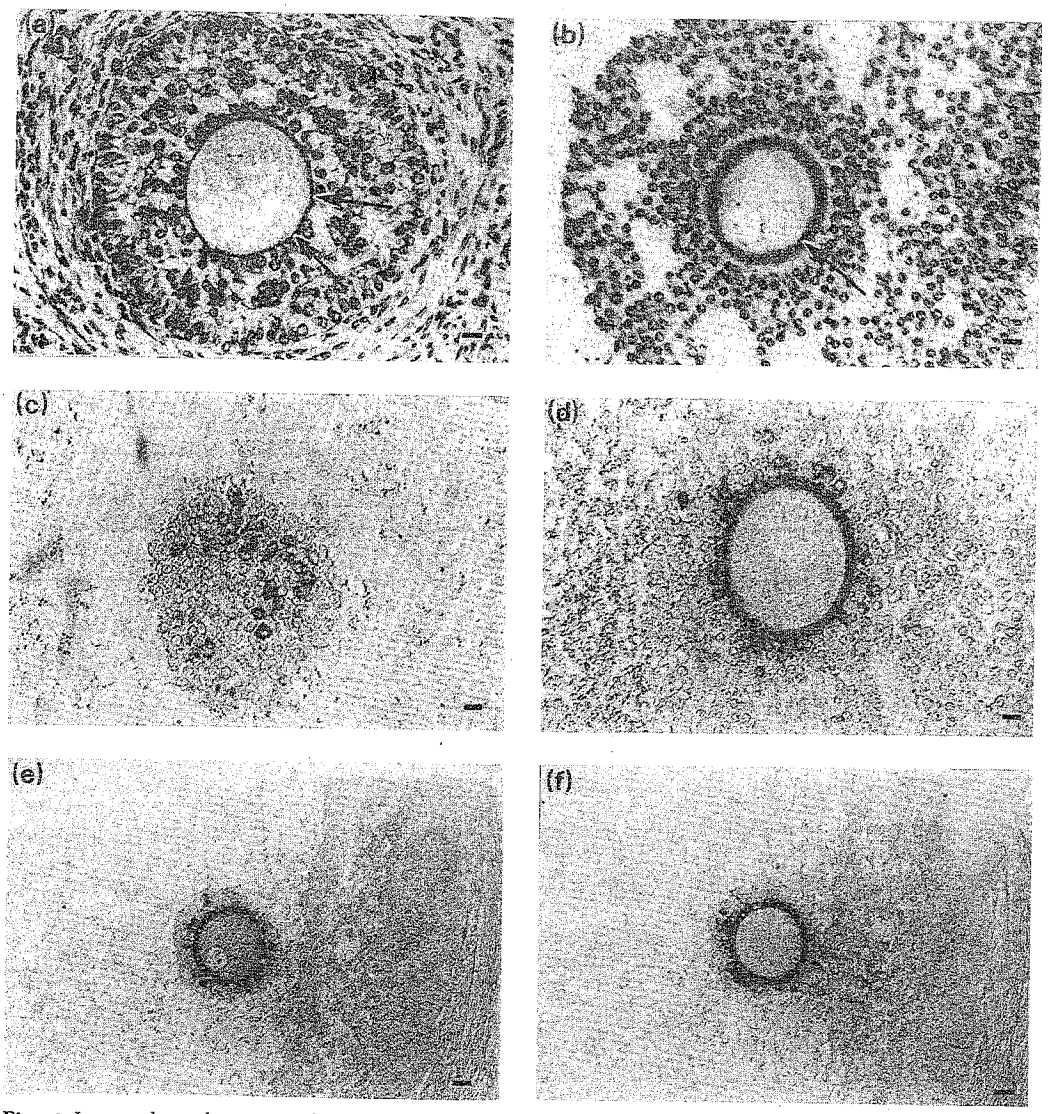

Fig. 4. Immunohistochemistry of sections (5 μm thick) of an adult deer ovary that has been embedded in paraffin wax and fixed with Bouin's fluid. Periodic acid Schiff (PAS) stain of (a) the zona pellucida (arrows) of a large secondary follicle, and (b) of the zona pellucida (arrow) within the cumulus mass of a large antral follicle. (c, d) Sections treated with guinea-pig anti-rabbit 55 kDa protein. (e) Section treated with guinea-pig anti-rabbit 45 kDa protein. (f) Section treated with guinea-pig anti-heat-solubilized rabbit zona pellucida. (Scale bar in a, b, c represents 30 μm; in c, d, e it represents 60 μm.)

antibodies used show a marked tendency to localize in oocyte cytoplasm during early growth and in a particular subset of granulosa cells from the earliest primary stage to the antral stage.

If sections probed with rabbit anti-deer zona pellucida antibodies (Fig. 5a, b) are compared with corresponding sections (Fig. 5f and e, respectively) probed with guinea-pig anti-HSRZ antibodies, an example of localization of antigenic determinants shared among species can be seen. Both probes share some similarities in localization patterns at the zona pellucida surfaces and in granulosa cells immediately surrounding the zona pellucida. However, the guinea-pig anti-HSRZ antibodies stain more deeply and over a greater area of the zona than do the rabbit anti-HSDZ antibodies, which appear to label only the inner and outer rims of the more mature zona pellucida (Fig. 5b) and populations of granulosa cells extending several layers further from the zona surface in both the secondary (Fig. 5a) and the antral (Fig. 5b) stages of follicular growth.

At the earliest follicular stage in which the zona begins to be elaborated (Skinner *et al.*, 1984; Wolgemuth *et al.*, 1984) the granulosa cells achieve a cuboidal morphology (Fig. 5c) and probing with the rabbit anti-HSDZ antibody results in deeper staining of the oocyte cytoplasm and labelling of some, but not all, of the new single layer of granulosa cells surrounding the oocyte. An eccentric section into a cumulus mass in an antral follicle shows the subpopulation of granulosa cells labelled by the rabbit anti-HSDZ antibody (Fig. 5d). This section may be compared with a corresponding section (Fig. 4c) probed with antibody against a major rabbit zona protein.

In all cases, several types of negative controls were run. Primary antibody solutions (serum as well as affinity-purified

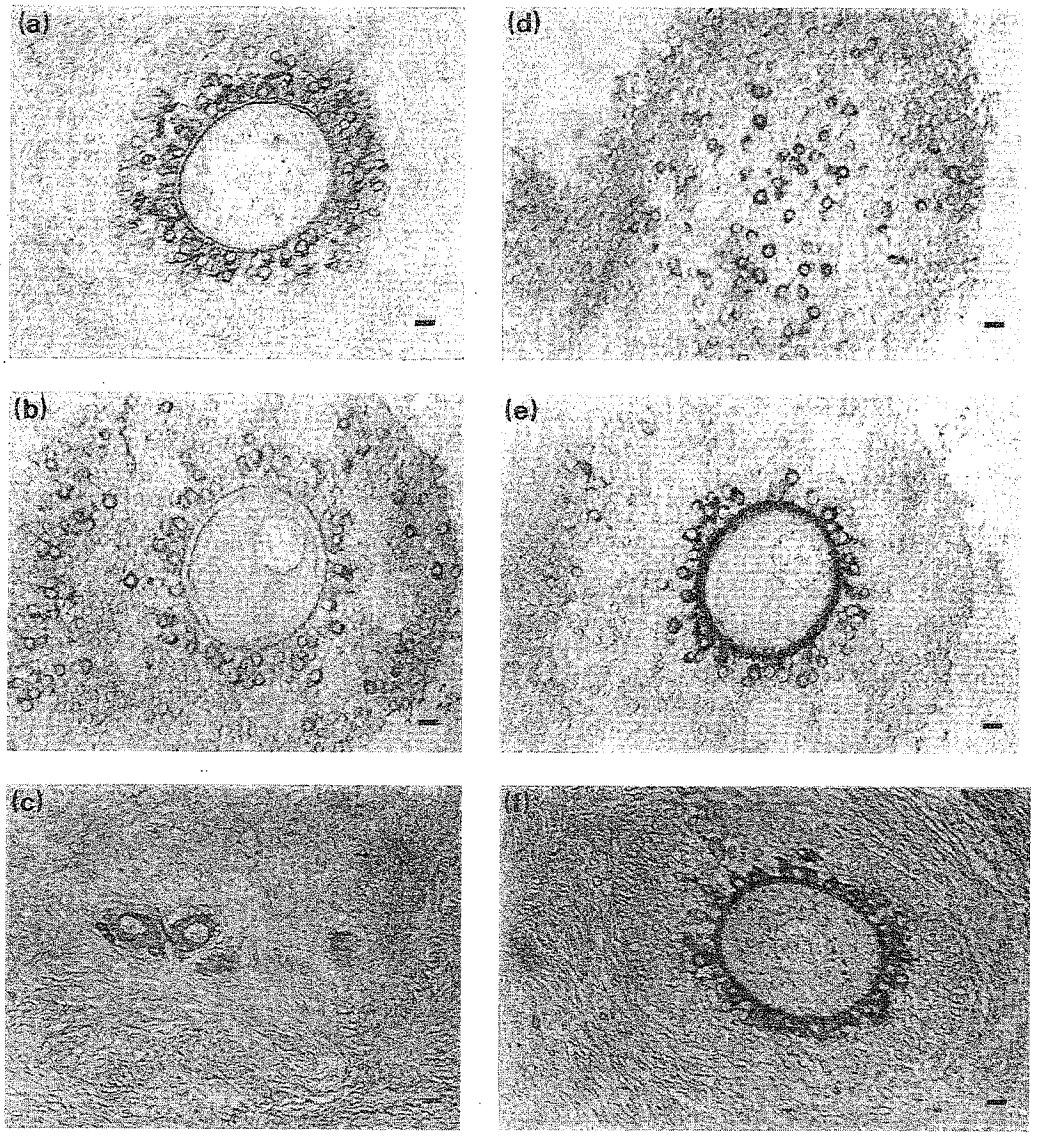

Fig. 5. (a, b, c, d) Follicles of adult deer ovaries probed with antiserum made in rabbits against heat-solubilized deer zonae pellucidae. Follicles range in stage from the earliest primary follicles (c) to secondary follicles (a) and antral follicles, depicted both in central cross-section (b) and in eccentric cross-section (d). In addition, corresponding sections (e, f) were probed with guinea-pig anti-heat-solubilized rabbit zona pellucida antiserum. (Scale bar represents 3 μm.)

antibody solutions) were run at increasing dilution until the nonspecific staining was absent or minimized, while the specific signal remained. At this same dilution, control antibody solutions were run side by side with primary antibodies on serial sections. In addition, negative controls consisting of secondary or tertiary antibody solutions and subsequent reagents were run on such sections. In no case was anything more than a light general background seen (not shown).

Discussion

Implementation of an immunocontraception strategy for management of a deer population requires the development of an immunogen with several critical properties. With respect to the use of immunogens based on zonae pellucidae, the molecules must be immunogenic and contain both T-cell and B-cell epitopes. The initial experiments reported here have established that deer immunized with pig zona pellucida glycoproteins will develop antibodies that recognize deer zonae pellucidae. Moreover, these same antibody populations recognize epitopes present in both pig and rabbit zonae pellucidae. Further studies demonstrate that epitopes of deer zonae pellucidae are recognized by anti-zona pellucida antibodies made in either rabbits or guinea-pigs. Collectively, these studies demonstrate that some zona pellucida epitopes are conserved among species.

The identification of shared antigenic determinants between or among species depends upon the choice of methods used to identify these epitopes. Several methods were therefore used

including (i) *in situ* localization fixation of antigens in immunocytochemistry studies, (ii) identification of protein antigens the structural integrity of which is maintained by noncovalent forces as in the heat-solubilized immunogens, (iii) identification of antigens associated with denatured or deglycosylated protein, and finally (iv) identification of more specific protein epitopes expressed in the recombinant zona proteins and protein fragments.

In addition to the evidence that these immunogens may be useful in population control strategies, these antibody probes have allowed us to characterize ovarian follicular development more thoroughly. The evidence presented here suggests that these zona pellucida molecules appear first in the oocyte cytoplasm and in some, but not all, of the early granulosa cells, as is observed in rabbits (Lee and Dunbar, 1993). With the growth of the follicle, these molecules localize into discrete bodies in the oocyte cytoplasm, into the growing zona itself and into a population of granulosa cells more distant to the zona pellucida. As the follicle approaches maturity these epitopes appear to segregate with certain granulosa cells into other areas of the follicle as they are not present in mature zona pellucida. These observations may result from modification of epitopes by fixative procedures or to reorganization of molecules in the matrix as the follicle grows. The significance of these changes throughout the growth of the follicle has yet to be determined, but may shed light on the age-dependent functioning of the granulosa cells and the cooperation between the oocyte and the granulosa cells in the formation and maturation of the growing zona pellucida. The manner in which mammalian ovarian follicular development proceeds and the degree of similarity with which this takes place in different species has important implications for future studies on ovarian development.

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